Do Toxic Equivalency Factors Predict Adverse Reproductive Effects of a Mixture of Dioxin and Dioxin-like Compounds

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related polyaromatic hydrocarbons (PAHs) have been shown to alter the reproductive development of laboratory animals [1,2,3]. While most laboratory studies have focused on the effects of individual compounds, this class of compounds exists as a complex mixture in the environment. To better estimate the toxicity of such mixtures, toxic equivalency factors (TEF), which define the toxicity of PAHs as a fraction of TCDD, have been developed. Therefore, to determine if the TEF system predicts adverse reproductive effects of mixtures, we used a dosing solution composed of a mixture of dioxins, furans and coplanar PCBs at relative concentrations that approximated the relative abundance of these compounds in a food mixture [4]. Following the work of Gray *et al.* [3] we dosed dams by oral gavage on gestation day 15 at doses of 0, 0.05, 0.2, 0.8, and 1.0 ug/kg toxic equivalency (TEQ). Dams were allowed to litter and the pups were monitored for a number of biological endpoints including body weight, anogenital distance, day of vaginal opening, day of preputial separation and the weights of reproductive organs. In addition, ethoxyresorufin-*O*-deethylase induction was compared between tissues derived from rats exposed to the TEQ mixture to tissues from animals exposed to TCDD only.

Materials and Methods

Chemicals. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin(TCDD), 2,3,7,8-tetrachlorodibenzo-*p*-furan(TCDF), 1,2,3,7,8-pentachlorodibenzofuran(1-PeCDF), 2,3,4,7,8-pentachlorodibenzofuran(4-PeCDF) and 1,2,3,4,5,6,7,8-octachlorodibenzofuran (OCDF) were purchased from Ultra Scientific (North Kingstown, RI; purity >98%). 3,3',4,4'-tetrachlorobiphenyl (PCB77), 3,3',4,4',5-pentachlorobiphenyl (PCB126), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB169) were purchased from Accustandard (New Haven, CT; purity >99%). For the preparation of dosing solution all chemicals were dissolved in acetone, diluted in corn oil (Sigma Chemical Co., St. Louis, MO) and the acetone removed by evaporation using a Savant SpeedVac(Savant Instruments Inc., Farmingdale, NY).

Animals. Time-pregnant Long Evans rats (gestational day 9/ day after mating= GD0) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Females were housed in plastic cages containing heat-treated pine shavings (Beta Chips, North Eastern Products Inc.,

Warrensburg, NY) and given food (Purina 5001 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*.

Dosing. Block one consisted of 45 dams of which equal numbers were dosed at 0, 0.8 or 1.0 ug/kg TEQ in corn oil at a dosing volume of 5ml/kg. The second block consisted of 45 dams of which equal numbers were dosed at 0, 0.05 or 0.2 ug/kg TEQ. In the third block, 75 dams were used with 15 dams dosed at 0, 0.05, 0.2, 0.8 or 1.0 ug/kg TEQ. All dams were treated by oral gavage on GD 15 using a dosing volume of 5 ml/kg.

Collection of Tissue for EROD Activity In order to compare ethoxyresorufin-*O*-deethylase activity of rats exposed to the TEQ mixture to TCDD alone, GD15 dams were dosed with 0, 0.05, 0.2, 0.8 or 1.0 ug/kg TCDD. Groups of 6 dams per dose per timepoint were sacrificed at GD21 or PND4. From each litter, maternal liver, or a pool of 4 fetal or pup livers or placentas, for GD21, were collected and processed according to the methods of DeVito *et al.* [5]. Briefly, tissues were homogenized in 10 volumes(w/v) of ice cold phosphate-buffered saline, pH 7.4 using 5-7 strokes of a glass-teflon homogenizer. Homogenates were centrifuged at 9,000xg for 20 min and the resulting supernatant was collected, snap frozen in liquid nitrogen and stored at -80°C for later analysis of EROD activity.

Collection of Tissue for Chemical Analysis Tissues were collected from dams and their offspring at GD16, GD21 and PND4. 6 dams per time point, per dose were dosed as above. On GD16 and GD21. Maternal tissues collected included serum, liver and adipose. Pup tissue collected included: all fetuses and placentas from GD16 litters and 1-2 fetuses or pups per litter on GD21 and PND4. These samples were transported to Triangle Laboratories, Inc. on dry ice for analysis by high resolution GC-MS.

EROD-Microsomal Preparation
S9 was removed from the freezer, allowed to thaw on ice and centrifuged at 100,000xg for 1hr. The resulting microsomal pellet was resuspended in 400 ml PBS and used for metabolism assays. Protein content of diluted microsomes was determined by the method of Bradford [6] using BioRad protein assay reagents (Richmond, CA) and a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Bovine serum albumin was used as the standard.

Animal care and Observation Dams were observed beginning on the morning of GD21 until all dams had littered. On the day following the birth of the pups (PND1) anogenital distance (AGD) and body weight were recorded for all pups. Litters were standardized by culling to 5 males and 3 females on postnatal day 4. AGD and body weights were subsequently recorded for all remaining pups on PND8. Body weight was also recorded on PNDs 15 and 22. Beginning on day 7, pups were checked daily for incisor eruption and from PND12 on for eye opening. Male pups were also checked for retained nipples. At weaning, animals were housed as above in unisexual groups of 2 to 3 rats/cage. Beginning on day 28, female pups were observed for vaginal opening and body weight recorded when opening was first observed. Similarly, male pups were observed from PND36 on for preputial separation and body weight recorded on the day of separation.

Animal Necropsis 1 male pup per litter was sacrificed on PND49 and 63 and necropsied. Whole body weight along with the weight of the liver, paired kidneys, paired adrenals, spleen, paired seminal vesicles with attached coagulating glands and their fluid content, ventral prostate, paired epididymes, and paired testis were recorded. The left cauda epididymis was removed from each animal, weighed and used to determined cauda epididymal sperm counts.

1 female pup per litter was sacrificed on PND70 and necropsied. The above organ weights recorded for male offspring were also recorded with the exception that in the female the reproductive organs weighed included the uterus and paired ovaries.

Sperm Counts For the determination of epididymal sperm counts the left cauda epididimis was removed, minced with a scapel blade and incubated in 2mls of phosphate buffered saline (pH 7.4) at 37°C for 15min. Following incubation, 0.1mls of 50% gluteraldehyde was added, the tube capped, vortexed and stored. Samples were counted using a hemocytometer following dilution (see below).

Statistics. Using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA), data was evaluated for statistical significance using one-way analyses of variance (ANOVA) followed by Fisher's PLSD test as a post hoc test. A $p<0.05$ defined statistically significant differences.

Results and Discussion

The range of doses employed in the current study did not prove to be overtly toxic to dams or their offspring. No changes in maternal weight gain during pregnancy or number of viable pups on PND1 were recorded. In the first round of exposures, significantly more male pups per litter were found at the highest dose, however, this finding was not repeated in the second exposure.

In the first round of exposures, the TEF mixture did not affect incisor eruption or eye opening. This observation is in contrast to reports for a similar TEQ administered dose of TCDD only in which eye opening was significantly advanced in pups exposed to 1ug/kg TCDD [1,3]. Incisor eruption was not affected by TCDD treatment [1].

Observations of preputial separation in male offspring and vaginal opening in females indicated that the TEF mixture caused dose-related increases in the time to puberty. In addition, females displayed a dose-response increase in the incidence of vaginal threads.

In contrast to effects on puberty, the TEF mixture did not cause substantial decreases in the weights of the sex accessory glands of the male reproductive tract reported for equivalent doses of TCDD alone. However, a preliminary data from males sampled on PND32 indicated that seminal vesicle weights were decreased at this time. At PND32 seminal vesicle weights show the greatest effect of TCDD exposure [1,7]. Therefore, pups from the second exposure are being sacrificed at PND32.

Chemical analysis of the dosing solutions indicated that the stock solution from which all dilutions were made contained lower than desired amounts of several compounds. This analysis suggests that the TEQ of the dosing solutions was 10-15% lower than planned. This observation may account for some differences between the TEQ mixture of the present study and equivalent

doses of TCDD alone. In addition, it is possible that pharmacokinetic differences between the compounds contained within our mixture and TCDD results in different TEQ concentration within target tissues at critical times in development. We are currently analyzing tissue samples for their chemical composition.

Conclusions

The use of TEF methodology appears to predict adverse reproductive effects of a mixture of Dioxin and Dioxin-like compounds. Determination of tissue concentrations of the chemicals used in this study will lead to a better interpretation of how closely the dose-response pattern for this mixture resembles the one for TCDD treated animals.

TABLE 1
PUBERTAL DELAY IN OFFSPRING EXPOSED TO TEQ MIXTURE *IN UTERO* AND LACTATIONALLY

TEQ DOSE	0	0.05	0.2	0.8	1.0
VAGINAL OPENING	31.26±0.2	31.7±.5	32.2±0.4	32.7±0.5	33.7±1.1[a]
PREPUTIAL SEPARATION	39.8±0.3	40.8±0.3	40.9±0.5[a]	42.2±0.7[a]	41.9±0.5[a]

a= significantly different from control $p<0.05$

TABLE 2
INCIDENCE OF VAGINAL THREAD FOLLOWING *IN UTERO* AND LACTATIONAL EXPOSURE TO THE TEQ MIXTURE

TEQ DOSE	0	0.05	0.2	0.8	1.0
INCIDENCE OF VAGINAL THREAD	9.5±5.2	25±9	22±11	58±21[a]	94±6[a,b,c]

a= significantly different from control $p<0.05$
b= significantly different from 0.05 $p<0.05$
c= significantly different from 0.2 $p<0.05$

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